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Price Three Dollars

The Proteins of Hevea brasiliensis Latex. Part 3. The Soluble Proteins of 'Bottom Fraction'*

G. F. J. MOIR and S. J. TATA

Paper electrophoresis has been used to examine aqueous extracts of the sedimentable material (bottom fraction) from fresh latex. A total of eight electrophoretically distinct protein components were observed in the extracts. Six of these were of general occurrence in bottom fraction from various different latices; the other two were detected sporadically.

The introduction by COOK AND SEKHAR (1953) of ultracentrifugation as a means of separating latex into its constituent phases has led to a number of investigations of the chemistry and enzymology of these phases. (ARCHER AND SEKHAR, 1955; ARCHER AND COCKBAIN, 1955; ARCHER, 1960; GASCOIGNE *et al.*, 1958; HSIA, 1958; MCMULLEN, 1959). In the first part of the present series, Archer and Sekhar described a study of the proteins of '*C* serum', the non-sedimentable aqueous phase of latex. This was found to contain a mixture of proteins which could be resolved into seven components by paper electrophoresis. We are now reporting an investigation by paper electrophoresis of the proteins of 'bottom fraction', the particulate material sedimented from latex by ultracentrifugation.

The published data on *C* serum (Archer and Sekhar) were obtained with freeze-dried samples prepared in this laboratory from the latex of mature seedling trees growing in Field 20A of the R.R.I. Experiment Station. Using freeze-dried bottom fraction from the same source, ARCHER (1960; and private communication) found that a mixture of proteins could be extracted from it with water or buffer solutions. Paper electrophoresis of such extracts showed two major components and suggested the presence of several minor ones as well.

We have used a technique of paper electrophoresis somewhat different from Archer's, have confirmed the presence of two major soluble protein components in freeze-dried bottom fraction and have obtained further information on the minor components. Our results include data on bottom fraction from various clonal latices as well as on material from seedling latex.

EXPERIMENTAL METHOD

Collection of Latex

Seedling latex was obtained from twenty mature mixed clonal seedlings in Field 20A of the R.R.I. Experiment Station. With a few exceptions, these were the same trees which had supplied latex for the earlier studies on proteins by Archer and his collaborators. They were tapped alternate daily on a half-spiral cut and the latex was collected into clean aluminium cups for 30–60 minutes after tapping. It was then pooled and brought to the laboratory.

Clonal latex was obtained from groups of mature buddings, each of 18 to 20 trees,

* The Natural Rubber Producers' Research Association and the Rubber Research Institute of Malaya have cooperated in research on latex proteins for some years. Other papers in the present series have appeared in the *Biochemical Journal, London*, as follows:

Part 1: ARCHER AND SEKHAR (1955)

Part 2: ARCHER AND COCKBAIN (1955)

Part 4: ARCHER (1960)

(see the list of references at the end of the present paper for further details).

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representing the clones Glenshiel 1 (Gl 1), RRIM 501, Tjirandji 1 (Tjir 1) and Pilmoor A44 (Pil A44). These trees were also tapped alternate daily whether or not samples were required for the work described here, i.e. the trees were in commercial tapping and not reserved for these experiments. In some cases tapping was not on a half spiral: details are given below. The latex was collected into clean aluminium cups for approximately one hour after tapping, then pooled and brought to the laboratory.

Isolation and Extraction of 'Bottom Fraction'

Latex samples were centrifuged in lusteroid tubes in Rotor No. 21 of a Spinco Model L ultracentrifuge. The rotor was accelerated as quickly as possible (about 20 minutes) to 20,000 r.p.m. (g(max) = 53,620) and this speed was maintained for 40 minutes; deceleration to rest occupied a further 20 minutes. Neither the latex nor the rotor was usually pre-chilled, but the refrigeration system of the centrifuge was operated during the run and slight cooling of the rotor and contents took place. (This procedure is substantially the same as was used in all the previous investigations cited at the beginning of this paper. It differs in detail from the 'fully refrigerated' centrifugation of latex mentioned by MOIR (1959), which is a more recent development). Separation of the latex occurred essentially as described by Cook and Sekhar. After the tubes had been pierced and the serum drained out, they were cut up and the surface of the gelatinous bottom fraction was lightly rinsed with distilled water. Bottom fractions from several tubes were then pooled, slurried with distilled water and the slurry freeze-dried.

Simple aqueous extracts of this freeze-dried material were used throughout the work: 0.5 g was extracted for 30 minutes, with stirring, at 0°C with 1.5 ml of distilled water. The paste obtained was then centrifuged at approximately 25°C for 30 minutes at 2,000 g (max) in an MSE centrifuge (Measuring and Scientific Equipment Ltd., London). Refrigerated centrifugation (0°C) did not seem to give a good separation of the aqueous extract from the insoluble residue. After centrifugation the extract was filtered before application to the paper strips used for electrophoresis. A turbidity passing the filter paper was occasionally observed. The total (Kjeldahl) nitrogen was determined in a very few extracts: figures of the order of 10 mg N per ml were obtained.

Electrophoresis

The apparatus employed the principle of DURRUM (1950) and was made locally from 'Perspex' sheet to a design freely adapted from that of FLYNN AND DE MAYO (1951). It is shown in Figure 1. The electrodes were mantles of platinum wire on glass frames in the earliest experiments; they were later replaced by carbon rods without observable effect on the results. The tanks were filled with barbiturate buffer, pH 8.6, μ = 0.05 (3.68 g of barbitone and 20.6 g of sodium barbitone in 2 litres of solution; 1 litre in each tank).

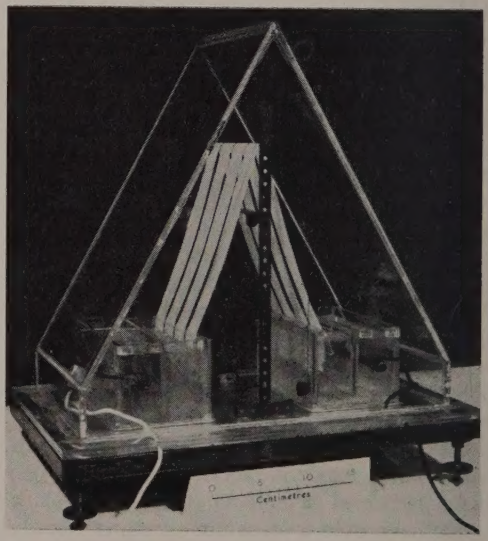

Figure 1. Apparatus for paper electrophoresis used in this study. The whole of the prismatic cover is removable.

Whatman No. 1 filter paper strips 3.1 cm (1¼ in.) wide and 50 cm long were used. Bottom fraction extract (0.01 ml) was applied across each of four dry strips at the centre, as a narrow band from edge to edge, by means of a micropipette. The strips were then placed in the apparatus with the centres resting on the horizontal glass rod at the apex, and the ends dipping into the buffer in the tanks. The effective length of paper, from one buffer surface to the other, was approximately 44 cm. Before the strips were mounted, the buffer was brought to the same level in each tank (siphon). The strips were then wetted with buffer on each side to within half an inch of the apex, the cover of the apparatus was placed in position and 90–120 minutes were allowed to elapse before the current was switched on. During this period the buffer fronts reached the apex, sharpening the initial band, but sometimes shifting it slightly. A constant current of 2 mA was then passed for 22 hours. The initial voltage across the electrodes varied but was generally in the range 195–245 V; this dropped to 140–180 V by the end of the run. Facilities for accurate temperature control were not available; the runs were performed in air-conditioned laboratories where the temperature was a few degrees below tropical room temperature.

At the end of the run the paper strips were immersed in 95% ethanol for 5 min. and then stained for 10 min. in a solution of 1% bromophenol blue in 95% ethanol saturated with mercuric chloride. Excess dye was washed off with 5% acetic acid and the strips were then dried in a horizontal position by a current of warm air and exposed to ammonia vapour before observation.

Some strips were treated with paraffin oil under partial vacuum in the usual way and passed through an EEL photoelectric scanner (Evans Electroselenium Ltd., Harlow, Essex). The curves obtained were of some use as records and for comparison with direct visual observations, but no quantitative significance was attached to them because it was obvious that the intensity of at least one of the bands on the finished electropherograms (component (viii), see below) varied uncontrollably with the time of washing in acetic acid.

Photography and Reproduction of Figures

The electropherograms were rarely if ever as clear as those obtainable with (for example) blood serum. Medium or low contrast photographs of the strips proved impossible to reproduce through the medium of a printing block. The blocks for Figures 3(a) to (g) were obtained after re-photographing the original photographs on a high contrast film (Kodak Microfile) and printing for maximum contrast. In some cases the figures exaggerate the clarity of the bands and in others slightly distort their relative intensities; their positions are, however, shown correctly.

RESULTS AND DISCUSSION

The procedures just described were developed using material from seedling (Field 20A) latex. The extracts proved fairly difficult to resolve; migration was rather limited even with the long time of run adopted. However, when the main details of the method had been worked out, it appeared that the proteins in the bottom fraction extract from this source could be resolved into two major components, with four fainter bands between them. This pattern of six bands, which is represented diagrammatically in Figure 2, recurred continually in the examination of further samples from the seedlings and from the four clones mentioned above. (See Figures 3 (a) to (g).) We have therefore found it convenient to interpret all results as repetitions of, or variations on this theme. The most important variation was the occasional appearance of two additional minor components; their positions are indicated by the arrows in Figure 2. For simplicity the bands are numbered from (i) to (viii) beginning with the band nearest the cathode end of the strip; with this system of nomenclature the fundamental pattern in our results is (i)-(iii)-(iv)-(v)-(vi)-(viii). All eight bands were never clearly seen together on a single freshly prepared electropherogram. Patterns showing the additional components as well as the fundamental ones were recorded as either (i)-

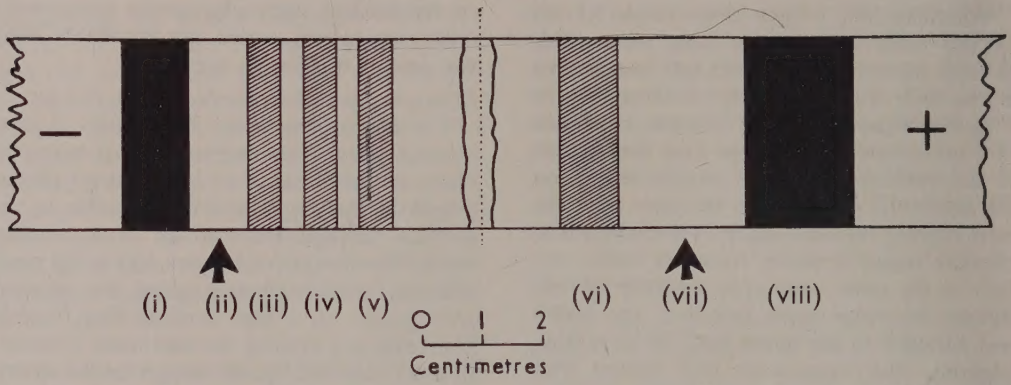

Figure 2. Diagram of stained paper electropherogram of bottom fraction extract, showing the basic pattern of six bands. The arrows indicate the positions of two additional bands of sporadic occurrence (see text).

(ii)-(iii)-(iv)-(v)-(vi)-(viii) or (i)-(iii)-(iv)-(v)-(vi)-(vii)-(viii). However, later re-examination of the strips suggested that some samples did in fact contain all eight components, one of the additional ones being very faint. See further below.

In Figure 2, the centre of the original strip, where the sample was applied, is represented by a dotted line. Around this position the final electropherograms always showed a stained smudge which is also indicated in Figure 2, and is visible in the photographs of Figure 3. It is due to insoluble material deposited at the origin during or shortly after application of the sample, and probably consists at least partially of denatured proteins. It is not, of course, due to a component of zero electrophoretic mobility since such a component would be expected to move away from the origin towards the cathode in consequence of electro-osmosis. We have neglected this band near the starting line, regarding it as 'false', but it is not impossible that a real band was present in some cases and concealed by the stained insoluble matter.

Using dextran we confirmed that electro-osmotic displacement was in the expected direction, cf. Archer and Sekhar, but no attempt was made to measure it or to determine the absolute mobilities of individual components. Our interest was, at this stage, mainly in resolving the samples, and a hanging strip apparatus such as ours is not suitable for mobility measurements. Even the apparent mobilities of the bands, i.e. their final distances from the origin, were not at all accurately reproducible: the distances indicated in Figure 2 are only rough approximations.

The major bands (i) and (viii) were observed without difficulty in samples from all sources (a partial exception was found only with some of the samples from Tjir 1, discussed below, in which the region around (i) appeared abnormal and was very difficult to interpret); some of the minor bands were often harder to see because of faintness, poor resolution or a general blurring of parts of the stained paper strip. Several fresh extracts were therefore prepared from almost all samples of freeze-dried bottom fraction, and each extract was also usually examined in quadruplicate (four strips). A number of electropherograms were thus available for all samples of bottom fraction and points of doubt were resolved as far as possible by comparisons between them. In some instances, when tests for polyphenol-oxidase or ultraviolet fluorescence were performed, some of the strips were not stained, but even in such cases at least three stained strips were prepared.

The electropherograms were always kept after observation, not discarded. When they were reammoniated after storage they sometimes seemed a little clearer than when quite fresh, and sometimes showed traces of minor components which had not been noticed earlier. This effect may be due in part to a slight overall fading with consequent reduction of background but there is of course a subjective element in the examination of paper electropherograms which cannot be eliminated in respect of the faintest bands. Thus a faint band whose presence is unexpected is likely to pass unnoticed, but to be revealed on careful re-examination of the same electropherogram. An example of this occurs in regard to component (vii) in some Tjir 1 samples (see below).

The aqueous extracts of bottom fraction from the seedling latex and from some of the clonal latices darkened during preparation and on the paper strip during the period of 90–120 minutes after application. This effect is ascribed to polyphenoloxidase activity. Hsia (1958) has shown the presence of the enzyme, accompanied by natural substrates, in bottom fraction; Chong (1956) has confirmed the presence of natural substrates and obtained evidence that they vary considerably in amount in different samples. The degree of darkening of a particular bottom fraction extract is thought to be related to the concentration of these (unidentified) substrates of the enzyme.

Quinones, including some which occur as intermediates in the enzymic oxidation of phenols, are well known to react with (tan) proteins. Hence a system of polyphenoloxidase plus substrate could, in principle, seriously modify proteins occurring in the same mixture. There is strong evidence that a reaction of this type occurs naturally in the cuticle of arthropods: for reviews of the extensive literature see Mason (1955) and Wigglesworth (1957). Cohen *et al.* (1956) have described an apparently similar effect in leaf extracts which contain soluble proteins and also display a browning reaction attributed to the presence of polyphenoloxidase and natural substrates. These authors developed an anaerobic extraction technique for leaves, which resulted in suppression of oxidase activity and thereby prevented any secondary attack on the other proteins in the extract. It was shown that such attack, if uncontrolled, discoloured some of the proteins and reduced their solubility.

It seems quite likely that similar reactions occurred in some of our bottom fraction extracts, with consequent damage to the soluble proteins. Such an effect is probably partly responsible for the 'false' band on the starting line of some of our electropherograms; we have, however, made no attempt to suppress it, and it appears that except in the case of one clone (Pil A44, q.v.) destruction of soluble proteins was not extensive enough to prevent their being resolved and observed.

The detailed results on bottom fraction from the various sources mentioned above are given below.

Figure 3. Photographs of stained paper electropherograms of bottom fraction extracts. Each photograph is the same size as the subject.

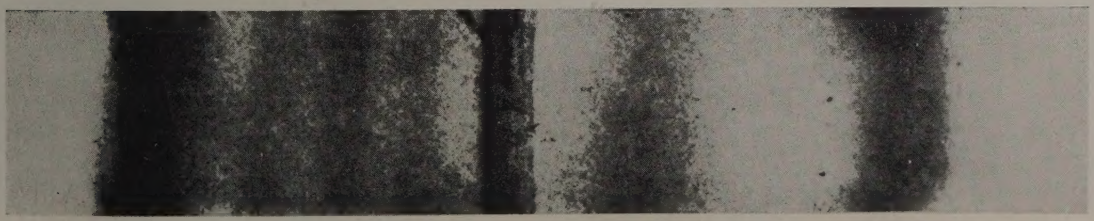

Figure 3 (a) Field 20A seedlings.

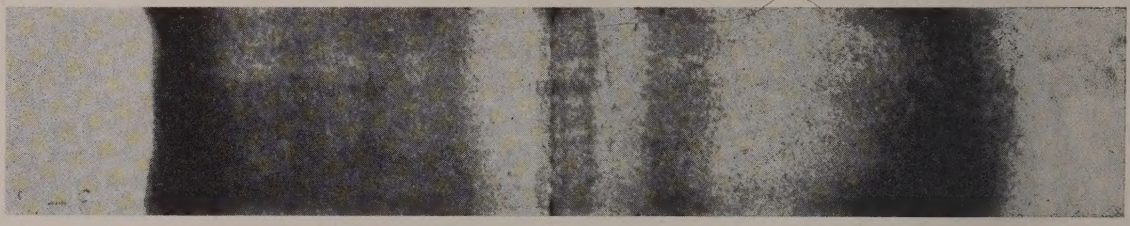

Figure 3 (b) Glenshiel 1.

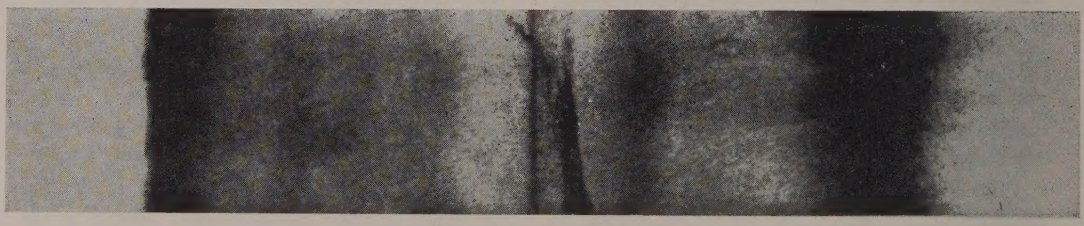

Figure 3 (c) RRIM 501

Figure 3 (d) RRIM 501 (another sample showing additional band, (vii); see text).

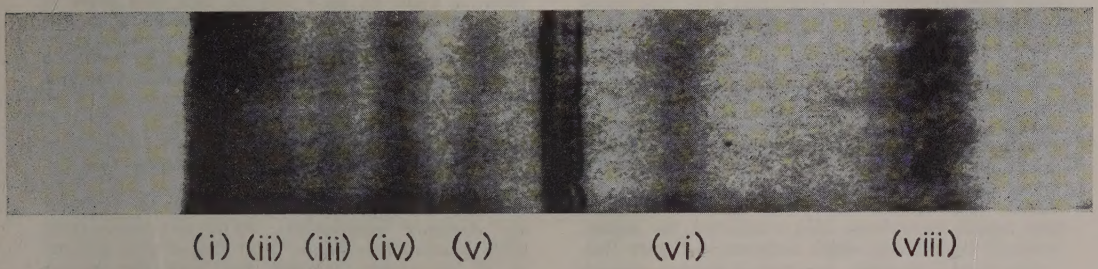

Figure 3 (e) Tjirandji 1.

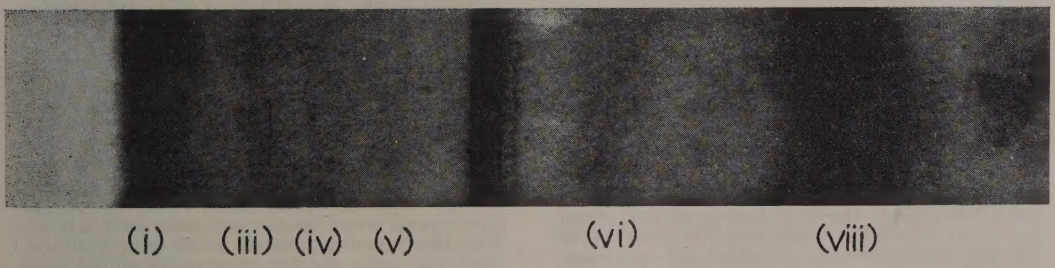

Figure 3 (f) Pilmoor A44.

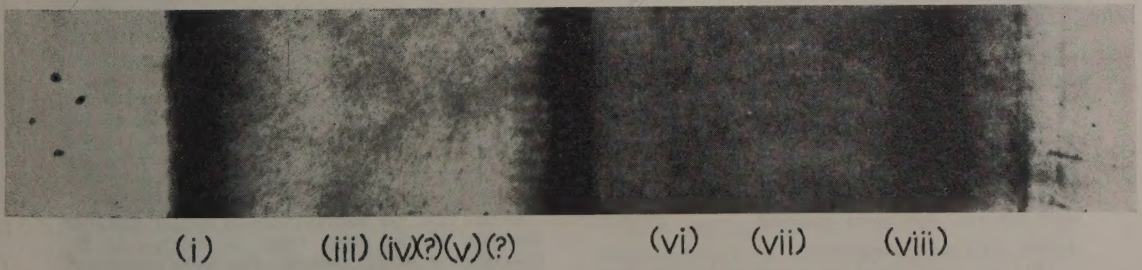

Figure 3 (g) Pilmoor A44 (another sample; see text).

Field 20A seedlings

As noted earlier, material from this source was used during the development of the experimental method, and the presence of the six components, (i)-(iii)-(iv)-(v)-(vi)-(viii) was detected before the procedures of extraction and electrophoresis described above had been standardised in all details. Six samples which were then examined by the standard procedure or by very slight modifications of it (application of extract to strips pre-wetted with buffer; centrifugation of extract at room temperature which was above 25°) confirmed the occurrence of this pattern, which is shown in Figure 3(a).

Two other samples were used in experiments which included alterations in the standard procedure not adopted in subsequent work (modifications in the design of the cover of the apparatus; longer times of run); these runs were performed at room temperature without air conditioning. Although non-standard, they supplied further confirmation of the occurrence of the six 'fundamental' components.

Component (vii), which was first detected in RRIM 501 material (see below), seemed to be present on some electropherograms of Field 20A material as well, but the band was extremely faint. This component probably occurs in bottom fraction from the seedling trees at a concentration near the level of detectability with the technique used. Also, the inner edge of band (i) was always diffuse and the appearance of the electropherograms in this region sometimes suggested the presence of component (ii); but it was never seen clearly as in the sample from Tjir 1 in which it was first noticed.

Glenshiel 1 (seven samples)

As with the seedlings, all trees used were tapped on a half spiral. The first sample of latex from this clone was taken from trees, in Field 18 of the Experiment Station, on which the cuts were approaching the union of stock and scion. The subsequent six samples all came from a different stand, in Field 48, and the cuts were in the middle region of the panel throughout, i.e. neither very new, nor close to the union of stock and scion. The aqueous extracts of bottom fraction from this clone did not darken on the paper strips but the extract did attack added catechol producing dark oxidation products. It was concluded that the untreated extracts did not darken because natural substrates of polyphenoloxidase were lacking, but that the enzyme itself was present.

The components (i)-(iii)-(iv)-(v)-(vi)-(viii) were plainly observed in all samples (except the third, in which (v) was not clear, though it was probably present). Figure 3(b) is representative. Neither component (ii) nor component (vii) was noticed on fresh electropherograms of Gl 1 material, but when re-examined after long storage some of the strips appeared to show component (vii) very faintly; the possible presence of component (ii) was also suggested as in the case of the seedling samples but a clear band (ii) was never observed.

RRIM 501 (seven samples)

All trees were tapped on a half spiral. The first sample of latex from this clone was taken from trees, in Field 30, on which the cuts were close to the union. Later a new half spiral panel was opened on the other side of the trees in the usual way. The second sample of latex was collected about six months after the new panel had been started and the remaining five samples over a period of eight months thereafter from the same panel.

Extracts of bottom fraction from this clone showed little or no tendency to darken on the paper strips.

When the first sample was examined only the six common components (i)-(iii)-(iv)-(v)-(vi)-(viii) were detected (see Figure 3(c)). These were present in all subsequent samples, but with the second sample the additional component (vii) was encountered for the first time and it recurred in samples three, four and five (see Figure 3(d) (from sample three)). This extra band was faint and in the case of sample four was definitely visible only after long storage of the electropherograms. With samples six and seven the band was extremely faint, though probably present.

Component (ii) did not appear clearly on any of the electropherograms of RRIM 501 material, but a few of the strips, including some on which band (vii) was visible, showed faint and doubtful indications of its presence.

Tjirandji 1 (Eight samples)

The first collection of latex from this clone was from a stand of trees, in Field 5, tapped on a half spiral, the cuts being already well down on the panel, roughly 6 inches from the union at the lower ends of the cuts. The aqueous extract of bottom fraction in this case was turbid even after filtration through Whatman No. 1 paper. It did not darken on the paper strips. Seven components were detected after electrophoresis, namely (i)-(ii)-(iii)-(iv)-(v)-(vi)-(viii), this being the first observation of the extra component, (ii) (see Figure 3(e)). Though not widely separated from component (i), the new band was quite unmistakable on almost all electropherograms from this sample (twenty-eight strips were prepared in this case).

Latex for samples two and three was taken from the old panels; bottom fraction from these again gave extracts which showed no darkening; component (ii) appeared to be present, in addition to the six common ones, but it was considerably more difficult to detect than before, and was seen on only a few of the strips; in the case of sample three, component (vi) was very faint and the region around it was blurred.

At this stage, five months from the beginning of experiments on Tjir 1, new panels were opened on the opposite sides of the trees. V cuts were used instead of half spirals, the bottom of the V being initially at about 85 inches from the union. The fourth sample of Tjir 1 latex was taken from these high cuts ten weeks after they were first opened.

In this case, the bottom fraction extract darkened distinctly on the paper strips, in contrast to the behaviour of earlier samples. The extra component (ii) was again clearly visible, as were components (i), (iii), (iv) and (viii). Components (v) and (vi)

were also present but faint and several of the strips were particularly difficult to interpret in the region around (vi), which was again blurred.

The remaining samples were all taken from the V cuts as these progressed down the tree. The tendency of the bottom fraction extract to darken appeared to lessen during this period; by the time a year had elapsed from the opening of the cuts the samples obtained showed only a slight browning reaction.

(This demonstration that polyphenoloxidase darkening occurs in samples from high cuts when it is less intense or absent with lower cuts on the same trees is novel only in respect of the method used. The tendency of whole latex from high cuts to darken has been known for over forty years (de Vries, 1920). See also next section (Pil A44)).

In sample five, components (iv), (v), (vi) and (viii) were all present, (v) being faint. The remainder of the patterns on the cathode side, i.e. the region expected to contain (i), (ii) and (iii), showed various abnormalities and was impossible to interpret with certainty, but all three components were probably present. Samples six and eight gave essentially the same result.

. Sample seven showed the six standard components (i)-(iii)-(iv)-(v)-(vi)-(viii) with no abnormalities except that band (iii) was faint. There was practically no evidence of (ii).

Component (vii) was not noticed on fresh electropherograms from Tjir 1 material, but when the strips were re-examined after long storage a considerable number of them (several strips from each of samples one, four, five, six and seven) were found to show this band very faintly. Since samples one and four also showed component (ii) clearly, it follows that these samples probably contained both 'additional' components as well as the 'fundamental' ones.

The tapping history of the Tjir 1 trees during collection of latex samples for these experiments appears rather unfortunate in retrospect, although it was in any case unavoidable at the time. More consistent results, in regard to component (ii), might perhaps have been obtained from a series of samples of latex collected from the middle region of half spiral panels.

Samples of latex from cuts very near the union of stock and scion (e.g. samples two and three in the present series) are objectionable on the ground that any truly clonal characteristics may disappear under the influence of the stock. Samples from high V cuts may have other peculiarities than the obvious browning reaction and may therefore not be strictly comparable with those from half spiral panels.

Pilmoor A44 (seven samples)

This clone was selected for study as a matter of curiosity because it has the reputation for producing a latex abnormally liable to spontaneous darkening. The tapping history was similar to that just given for Tjir 1, the Pil A44 trees used being also in Field 5.

The first collection of latex was made from the lower part of a half-spiral panel. The bottom fraction extract showed particularly strong darkening during preparation and after application to the paper strips. Nevertheless the six common components were detectable on the final electropherograms. See Figure 3(f). When these were kept for some time faint indications of the presence of component (vii) were seen (barely visible in this photograph).

The trees were now tapped on new V cuts at about 85 inches above the union. The second sample of Pil A44 latex was taken from these cuts three weeks after opening. Bottom fraction extract prepared from it darkened even more strongly than in the case of the first sample. The electropherograms showed a strong band on the starting line and the overall appearance was obscure. The major components (i) and (viii) were clearly visible, as was component (vi), but in place of the three components (iii)-(iv)-(v) only two faint bands were seen, and their identity was uncertain. There was a slight suggestion of the presence of component (vii).

Five further samples from the continuation of the same panel gave similar results: the darkening reaction was very strong and the electropherograms were all somewhat obscure, though components (i), (vi) and (viii) were obviously present. The region where components (iii)-(iv)-(v) were expected was blurred and faintly stained; with three samples two bands appeared to be present here; with the remaining two samples the region appeared to contain three bands blurred together; in all cases the identity of these bands was uncertain. The extra anionic component (vii) was detectable, though also rather blurred in all of the last five samples. Figure 3(g), from sample seven, is representative.

In the Pil A44 samples only very faint and uncertain indications of component (ii) were seen.

As suggested earlier in this discussion the obscurity of the electropherograms from Pil A44 material is probably connected with the intense browning reaction which occurs in this case.

It will be obvious from the striking resemblance between the electropherograms from numerous samples why we have chosen to interpret our results as explained at the beginning of this discussion and in Figure 2. This resemblance cannot be meaningless; the interpretation adopted provides a convenient nomenclature for bottom fraction proteins, and the paper electrophoretic procedure has immediate application in experiments on their fractionation and purification.

It is recognised that in the absence of measurements of mobilities the definitions of the 'components' are quite loose; conse-

quently it is not certain that (for example) the protein of band (iii) in a Gl 1 sample is fully identical with the one similarly designated in Tjir 1, and it is not possible as yet to establish a relationship between the eight components from bottom fraction and the seven reported in latex serum by Archer and Sekhar. Also, the components (i) to (viii) may not be homomolecular proteins but complexes of molecules of similar mobility which could be separated by more potent techniques. Similar considerations are now well known to apply to the proteins of blood serum.

An attack on these problems requires the characterisation of the components, and hence their separation on a preparative scale. Archer and Cockbain (1955) and Archer (1960) have published work of this kind carried out by classical methods of protein fractionation. In this laboratory we are studying ion exchange chromatography with cellulose derivatives (Peterson and Sober, 1956; Sober *et al.*, 1956) as a means of preparation of individual proteins from bottom fraction.

However, some incidental observations were also made during the paper electrophoretic work which bear upon the characterisation of some of the components and are therefore of interest here.

Stained electropherograms viewed under ultraviolet light showed a fluorescent band in the position of component (i). This was also evident with strips dried at about 100°C immediately after removal from the apparatus, washed with aqueous ethanol to remove buffer salts, redried and compared with strips from the same set stained in the ordinary way. Such fixed but unstained strips showed a fluorescent band in the position of component (i) with starting material from Field 20A seedlings, and each of the four clones. This suggests that component (i) is itself fluorescent.

Component (viii) was usually somewhat diffuse and tended to wash off the strips after staining, if the washing in 5% acetic acid was prolonged. This effect seemed to occur with samples from all sources, and was very much less marked with the other components, (i) to (vii).

When strips which had not been heated or fixed in alcohol were treated with dilute catechol solution immediately after electrophoresis, a rapid browning was produced in the region occupied by components (iii), (iv), (v), suggesting that the enzyme polyphenoloxidase is associated with all three bands. This effect was observed with Field 20A seedling material; samples from other sources were not fully tested but some evidence was obtained that the same association occurs in Gl 1.

Differences in the protein complement of individual organisms within a single animal species have been demonstrated by Smithies (1955) and Ashton (1957) using starch gel electrophoresis. It seems quite likely that analogous differences would occur in the proteins of different clones of a single plant species, for example in the latex proteins of different clones of *Hevea brasiliensis*. A report has already appeared in the literature (Balai Penjelidikan dan Pemakaian Karet, 1958) that 'difference in the protein patterns of the clonal latices Tjir 16 and PB 186 could be shown' (by paper electrophoresis), but details of this work do not appear to have been published.

The results of the present investigation give some slight evidence in favour of this idea: component (ii) was detected clearly only in material from Tjir 1. However, the technique we have used is not competent to establish intraspecific differences definitely. Furthermore, in our experiments the effects of variations in methods of tapping, in the positions of the cuts on the trees and in the season at which samples were collected are difficult to disentangle from any truly clonal peculiarities of bottom fraction proteins which may occur. Some of these complications, notably the effect of season, would be impossible to eliminate with any plant material. Nevertheless it is of interest to apply a more refined electrophoretic technique to latex proteins for several reasons, and starch gel electrophoresis is now

under study in our laboratories. The results will be reported subsequently, but it may be noted that it is already possible to distinguish a larger number of proteins in both serum and bottom fraction than have been detected by paper electrophoresis.

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VOLUME 16 PART 4

Detection and Estimation of Growth Substances in Hevea Latex

S. G. BOATMAN

Methods are described for the extraction, chromatography and bio-assay of plant growth substances in Hevea latex. There is evidence for the occurrence of at least two, and probably three active compounds in the extracts, but the commonly-occurring auxin, 3-indolylacetic acid (IAA), does not appear to be present in detectable amounts.

CHAPMAN (1951) has described a technique for the extraction of natural hormones from serum of *Hevea* latex, the growth substance activity of the extracts being determined by the classic *Avena* curvature method. He noted that the curvature obtained was not proportional to extract concentration and that the maximum response varied with the source of latex, never being as high as that induced by optimal concentrations of 3-indolylacetic acid (IAA). Chapman was thus led to believe that he was dealing with a mixture of active compounds. Nevertheless, he was able to demonstrate considerable variations in hormone content and to suggest that a relationship existed between latex yield and hormone level.

Since Chapman's work, the use of synthetic growth substances such as 2,4-dichlorophenoxyacetic acid (2,4–D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5–T) to stimulate latex flow, pioneered by CHAPMAN himself and by BAPTIST AND DE JONGE (1955), has become an accepted part of Malayan estate practice. The success of these yield stimulant mixtures has led to renewed interest in the possible presence, within the tree, of naturally-occurring substances which might themselves influence latex yields. The present paper describes the application of paper chromatographic methods in a re-examination of the problem.

MATERIALS AND METHODS

Reagents

All reagents were of Analytical Reagent grade. Water was glass-distilled.

Latex Collection

Latex samples employed in the preliminary studies reported here were taken from a compact group of twenty-eight-year-old clonal seedling trees tapped alternate daily on a half-spiral cut in good renewed bark. The group contained trees of a wide range of yield capacity with a mean yield of approximately 25 grams dry rubber per tree per tapping. Latex was collected from all trees in the group, mixed thoroughly and sieved to remove bark fragments. Aliquot samples were then taken for extraction. The extraction process was started within two and a half hours of tapping.

Extraction of Latex

An essential preliminary to any extraction procedure is the removal of the rubber hydrocarbon. Chapman achieved this by coagulation with phosphoric acid at 40°C, followed by extraction of the expressed serum. During the course of the present investigation comparisons were made between coagulation and extraction in cold ethanol and various acidic coagulation procedures and it was invariably

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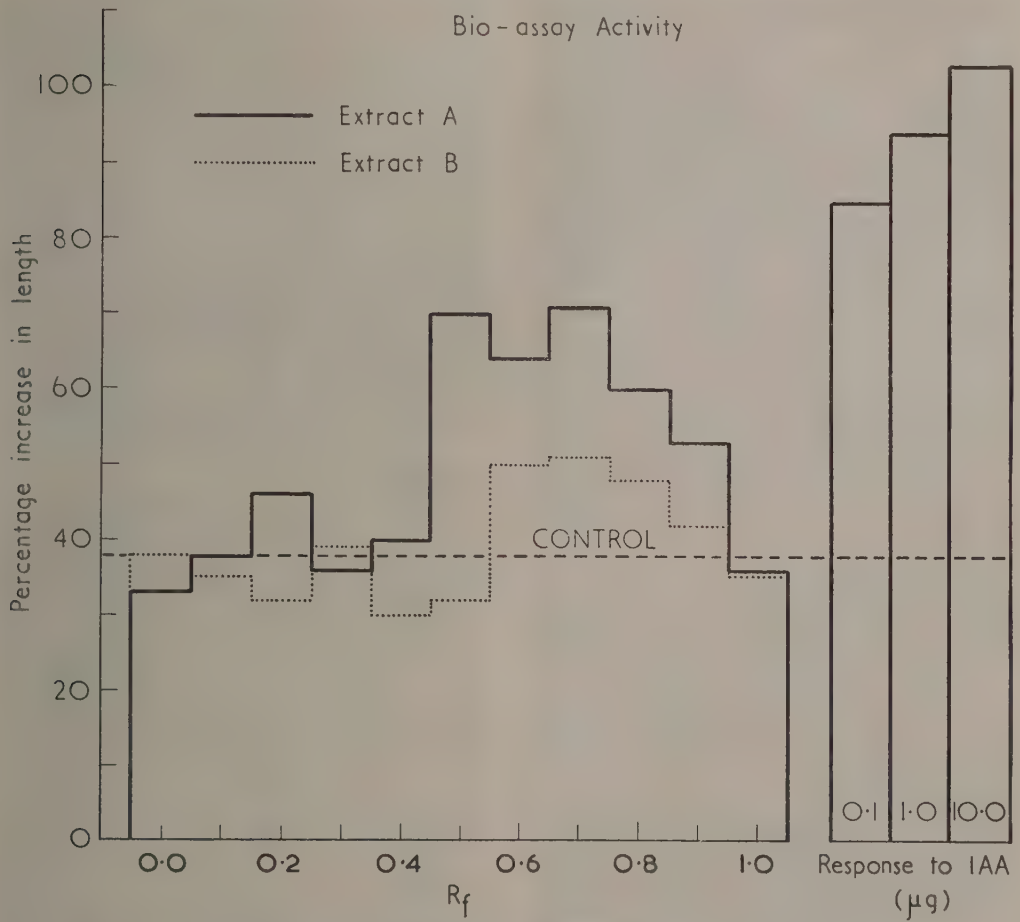

Figure 1. Comparison of the activity of chromatograms of two ether extracts of Hevea latex. Extract A was made by the alcoholic coagulation process described in the text, while Extract B was prepared by acidic coagulation followed by ether extraction of the expressed serum.

found that acidic coagulation resulted in an extract of lower bio-assay activity. Thus Figure 1 illustrates a comparison of extracts prepared by alcoholic coagulation, followed by ether extraction at a later stage (as described below) and by coagulation with phosphoric acid at 40°C and extraction of the serum with ether. The higher activity of the first extract is evident. It was noted that there was little or no sign of enzymic darkening in the case of extracts prepared by the use of ethanol, whereas this was marked in those made following coagulation with acid.

Latex samples of 250 ml were normally employed, although larger or smaller samples could readily be handled with a suitable adjustment of the quantities of reagents.

Standardised procedure. The latex sample was slowly mixed with an equal volume of cold ethanol with constant stirring, the hydro-

carbon coagulating almost at once. The sample was stored at –15°C for approximately 24 hours. (Longer or shorter periods of extraction at this low temperature had little apparent effect on the activity of the extract). The extract was squeezed through muslin or similar cloth, filtered and the alcohol evaporated under reduced pressure. It was found that further extraction of the rubber coagulum with fresh ethanol yielded only a negligible amount of additional activity and this procedure was therefore omitted. The aqueous residue after evaporation of the alcohol was made up to 150 ml with distilled water, adjusted to pH 2.0 with phosphoric acid, and extracted four times with peroxide-free ether. The ether extract was washed once to remove acid, dried over anhydrous sodium sulphate, and evaporated to dryness under a stream of nitrogen.

Paper Chromatography

Extracts were streaked along the starting line of a sheet of Whatman No. 1 paper (18 × 20 inches). A concentration equivalent to 20 ml latex per cm was found convenient. Two extracts could be compared on a single sheet, together with standard spots of 10 μg each of 3-indolylacetic acid (IAA) and 3-indolylacetonitrile (IAN). The solvent employed was *n*-butanol: ammonia (0.880) : water (100: 3 : 18 by volume). Operations were carried out in an air-conditioned laboratory with extreme temperature fluctuations of 22 – 27°C. The variation in temperature on any one occasion never exceeded 2°C. After equilibration for eight hours, the chromatograms were developed overnight by the descending method until the solvent front had travelled some 25 cm. The position of the solvent front was marked and the papers dried in a stream of air.

Preliminary Examination and Subdivision of Chromatograms

The dry chromatograms were examined under ultra-violet light and the positions of fluorescent areas lightly pencilled in and their descriptions noted. A strip 2.5 cm in width was taken from the centre of the chromatogram for bio-assay. This strip was subdivided into eleven equal portions of mean R_f 0.0 (starting line), 0.1, 0.2, etc., up to R_f 1.0 (the solvent front). In some of the earlier experiments the chromatogram strips were subdivided according to the u.v. fluorescence pattern rather than by R_f, but this proved unsatisfactory because there was no strict correlation between activity and fluorescence and because comparison between chromatograms was difficult owing to uneven division. Each paper square was placed in an appropriately-labelled bio-assay dish.

Parallel strips from each chromatogram were taken for test with chromogenic reagents.

Bio-Assay

Ideally, before any conclusions are reached concerning the physiological role of a compound in a particular plant, it should be tested on the plant itself under conditions as similar as possible to those obtaining in nature. Thus in the present investigation, if we are interested in the presence of natural compounds in *Hevea* capable of influencing latex flow after tapping, we should demonstrate their effect on that process. Unfortunately the amounts of active material separated on our chromatograms would be quite inadequate to produce any effect when applied to the rubber tree as a yield stimulant (as much as 0.1 g of active material is required for each tree in normal yield stimulation experiments). Even if such a procedure were practicable, it would be extremely cumbersome and time-consuming. Nor has any laboratory test method been devised which employs *Hevea* tissues and which gives a reliable estimate of the effect of a given compound on latex flow.

In these circumstances, it was decided to employ one of the conventional growth substance assays, the *Avena* coleoptile straight-growth test. It is known from a study of a wide range of synthetic compounds (BLACKMAN *et al.*, 1960), that activity in this test is reasonably well correlated with yield-stimulating ability. It is also well-suited to routine

laboratory use. The straight-growth test was preferred to the *Avena* curvature test employed by Chapman, partly because of the relative simplicity of the techniques employed and partly because a number of synthetic compounds which are highly active yield stimulants, such as 2,4–D, are inactive in the curvature test.

The procedure adopted was as follows: Oat seeds (variety 'Victory' from Svalof) were husked and soaked in water for two hours at room temperature. All subsequent operations were carried out in a dark room maintained at 25 ± 1°C. The seeds were sown, embryos uppermost, on several layers of water-saturated paper tissue contained in half glass bricks. The upper half of each glass brick was then placed in position, maintaining a suitable high humidity. For the first 48 hours the seeds were submitted to low intensity red light to suppress mesocotyl growth. Thereafter they were grown in darkness and handling operations were conducted under dim illumination from tungsten lamps filtered through Kodak OB yellow-green filters.

When the coleoptiles had reached some 2.5 cm in length (the time taken for this stage to be reached varied widely with seeds from different harvests and with different storage conditions), those of uniform length were selected and a 5 mm section cut 3 mm from the tip of each coleoptile with a suitable cutting device. The primary leaves were allowed to remain within the sections which were then floated for three hours on a dilute solution of manganese sulphate (1 mg $MnSO_4.H_2O$ per litre).

Ten sections were placed in a small covered culture dish containing one of the paper segments from the chromatogram and 1 ml of 2% sucrose solution adjusted to pH 5.0 with potassium phosphate/citrate buffer (10^{-2} *M* K_2HPO_4). Initially, 'blank' chromatograms were developed and bio-assayed for control purposes. Since the results so obtained did not differ significantly from those using only a single blank square of filter paper, the latter procedure was adopted for the sake of simplicity.

In addition to the control, standards of 0.1, 1.0 and 10.0 μg IAA were spotted on filter paper squares for bio-assay with each batch of chromatograms. It was not usually practicable to perform duplicate assays on the chromatograms, but wherever possible the controls and standards were replicated to give a measure of variation between dishes.

It was found that straighter growth was obtained by gentle agitation of the dishes on an oscillating table. After about twenty hours, the sections were removed from each dish in turn, blotted lightly, placed on a glass slide in a photographic enlarger and the length of the images measured at × 4 magnification using a transparent plastic ruler. The mean lengths were expressed as a percentage of the original section length and plotted as a histogram against R_f on the chromatogram. The control sections normally increased in length by some 40%. Significant increases over this control growth were attributed to the presence of growth-promoting substances.

Spray Reagents

The following chromogenic reagents were employed:

Modified Ehrlich's reagent. A 1% solution of *p*-dimethyl-amino-benzaldehyde in ethanol, mixed with an equal volume of concentrated hydrochloric acid immediately before use.

Ferric/perchloric reagent. Five per cent perchloric acid and 0.05*M* ferric chloride solution mixed in the proportions 50 : 1 by volume just prior to use.

Nitrite/nitric. Equal volumes of concentrated nitric acid and 0.5% aqueous sodium nitrite mixed immediately before spraying.

RESULTS

A typical histogram obtained from a chromatogram of the latex extract is shown in Figure 2. It will be seen that the principal active area lies between R_f 0.6 and R_f 0.9

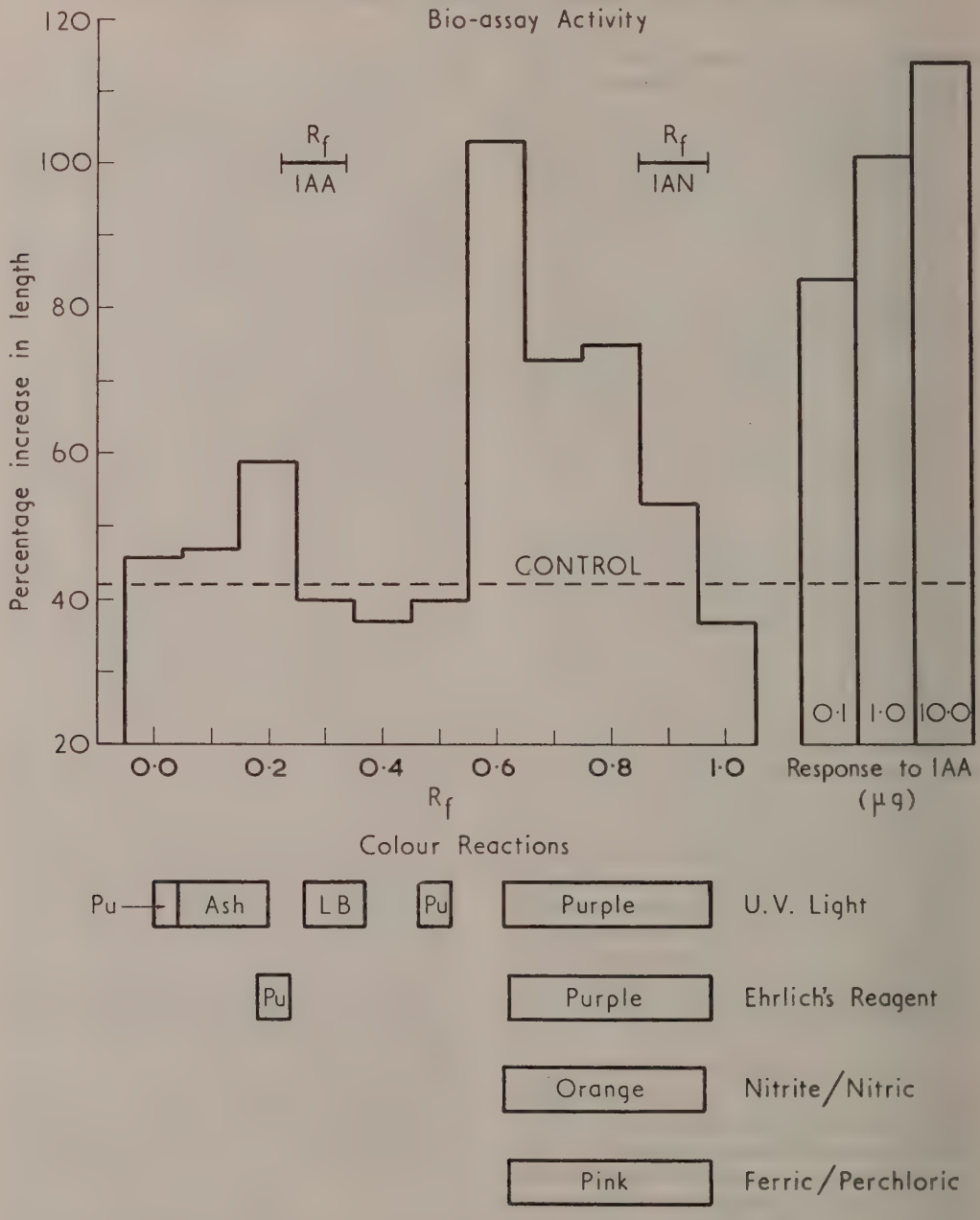

Figure 2. Histogram of bio-assay activity along the length of a chromatogram of a latex extract, compared with controls in sucrose-buffer alone and with the addition of known amounts of IAA. Colour reactions obtained on parallel strips of the chromatogram are also shown, drawn to the same Rf scale.

and that this is accompanied by strong colour reactions with indole reagents. These colour reactions differ from those of IAN (Ehrlich's ash, ferric/perchloric faint buff and nitrite/nitric dark blue) and other synthetic indole compounds of similar R_f (Sen and Leopold, 1954). In certain chromatograms there is a suggestion of a partial separation of the active zone into two, with peaks at R_f 0.6 and 0.8. It has also been noted that there is little correlation between the intensity of the chromogenic reactions in different chromatograms and the degree of bio-assay activity recorded. It is thus thought likely that we are dealing with at least two compounds in this region of the chromatograms. It is hoped that further investigations, employing other solvent systems, will provide more evidence on this point.

The slight activity found at about R_f 0.2 and associated with a purple Ehrlich's reaction, was thought at first to be due to a small amount of IAA (whose R_f in the standard spot is only slightly higher). However the colour reactions with Ehrlich's reagent are not identical, the unknown compound giving an immediate purple colouration as opposed to the blue colour formed by IAA on warming or standing. No colour reactions were observed with the other sprays until larger quantities were chromatographed, when a faint ash colour was obtained with ferric/perchloric and a yellowish colouration with the nitrite/nitric reagent. IAA (1 μg or less) gave a pink and a red colouration, respectively, with these two sprays under identical conditions.

In another experiment, synthetic IAA was added to latex before extraction (125 μg in 250 ml). The IAA was readily detected in the resulting chromatograms, giving typical colour reactions. A partial separation (as judged by their differing colour reactions) was achieved between the IAA and the naturally-occurring compound, the latter having a somewhat lower R_f.

It was therefore concluded that the unknown compound was not IAA and that the latter substance was not present in detectable amounts.

Some colour reactions were often noted in the initial streak (R_f 0.0) and these were occasionally accompanied by slight bio-assay activity.

DISCUSSION

The techniques described provide a comparatively simple method for the comparison of the growth substance content of latices from different sources. Work employing these methods to investigate growth substance levels in trees of varying yield capacity and following yield stimulation with synthetic compounds is in progress and will be reported elsewhere.

Like most methods employed for auxin estimation (Bentley, 1958), the work reported here was confined to ether-soluble substances. It is at present being supplemented by an investigation of ether-insoluble material, the results of which will also be described in a further publication.

The presence of IAA in *Hevea* leaves has been reported by Bolle-Jones (1954) and it was naturally anticipated that this apparently ubiquitous plant auxin would also be found in latex. However, no trace of it has been detected in the latex samples examined to date, despite the use of a variety of extraction procedures. When IAA was artificially added to latex before extraction it was readily recovered and identified, thus confirming that the methods employed were adequate for its detection.

A few experiments, of a preliminary nature, also failed to detect IAA in extracts of *Hevea* leaves at various stages of development. While the possibility of the occurrence of free IAA in *Hevea* tissues cannot be ruled out, it does appear possible that IAA was liberated from other indole compounds during the extraction procedure employed by Bolle-Jones.

As will be seen from the review by Bentley, cited earlier, the absence of detectable amounts of IAA has already been reported for a number of plant tissues. It appears possible that IAA may not be the auxin

operative in certain tissues, at least in some stages of their development.

Similarly, there have been reports of the detection of unidentified growth-promoting substances, some giving indole-type reactions, from a wide range of plant tissues. When further knowledge has been accumulated, it would be of interest to observe whether the *Hevea* auxins can be equated with any of those isolated from other plants.

In conclusion, it should again be emphasised that the activity of these naturally-occurring compounds in the *Avena* coleoptile straight-growth test constitutes no proof of their activity on latex flow, despite the general correlation between the activity of synthetic compounds in the two systems. Reasonable certainty on this point could only be achieved by the isolation of very much larger amounts of material, permitting tests on the tree itself.

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Loss of Viability of Three Root Parasites in Infected Root Sections Buried in Soil

K. P. JOHN

An account is given of observations carried out over a period of several years on the viability of the three major root diseases of Hevea in sections of buried roots.

The rubber tree becomes infected with root disease by vegetative growth of the fungus from diseased material in soil, most usually by root-to-root contact. It is well known that the root parasites can remain viable in large stumps for several years, but there are no data for fragments of diseased roots buried in soil—a point of considerable importance in relation to root disease control in replanting, particularly when clearing by mechanical methods, which leave diseased root fragments widely distributed in the soil.

Reference has already been made in the annual reports of the Institute (NEWSAM, 1953; ALTSON, 1953a and 1953b) to an experiment laid down to investigate the survival of root disease fungi in buried root sections. A full account of the experiment is now presented, with related observations from later experiments.

EXPERIMENTAL METHODS

An area of about 1000 sq. ft in Field 37 on the R.R.I.M. Experiment Station was thoroughly dug over to a depth of two feet and all buried timber and roots removed. The soil (a sandy loam) was kept clean-weeded throughout, and in it, roots infected with each of the three major root diseases—*Fomes lignosus* Klotzsch, *Fomes noxius* Corner and *Ganoderma pseudoferreum* (Wakef.), van O. et St.—were buried as material became available. The root sections were placed at a depth of one foot, arranged in six groups according to thickness and into two groups according to length, as shown in Table 1. The roots selected for use were well penetrated by the pathogen, yet still hard; those which had reached the advanced state of decay known as soft rot were rejected.

Enough material was buried to allow for sampling of the smaller roots over a period of two years and of the larger roots over four years. A total of 1296 root sections were buried.

Pairs of roots of each size class and for each pathogen were removed for examination

TABLE 1. APPROXIMATE VOLUMES OF ROOT SECTIONS BURIED (CUBIC INCHES)

Length of root, inches	Diameter of root, in inches:					
	⅛	¼	½	1	2	3
12	0.2	0.6	2.4	9.4	37	85
24	0.4	1.2	4.7	19	75	170

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at intervals of two months, the fungus being recorded as viable if it could be seen growing on the surface of the root or be isolated from within it. It does not follow that a root section thus classified as containing a viable fungus could still be infective.

RESULTS

The results, presented in Figure 1, show that the rate of loss of viability was inversely related to the size of the root, and that it varied relatively little with the different parasites. The difference in rate of loss of viability between *F. noxius* and *F. lignosus* which had been apparent after twelve months burial, was less evident after two years. *G. pseudoferreum* was the most persistent of the three parasites.

In the process of disintegration of roots, particularly those infected with *F. lignosus* and *F. noxius*, the wood rots away first, the bark remaining firm for a few months longer. Fresh growth of the fungus was often found on the surface of such persisting bark even after the entire wood had rotted away.

Observations on the Loss of Viability of F. lignosus from Other Sources

Inoculation experiments with *F. lignosus* subsequently carried out elsewhere on the R.R.I.M. Experiment Station gave further opportunities to study the behaviour of naturally infected root sections, in the presence of roots of rubber and cover plants.

In his first inoculation experiment with *F. lignosus*, JOHN (1958) found that root sections used as inocula, ranging in size from about 12 to 61 cubic inches, were partially or completely disintegrated after being in the soil for one year, the pathogen being no longer viable.

In the second inoculation experiment (initially designed to test the suitability of various fungicides for the prevention of superficial growth of *F. lignosus* on roots), the sections of infected root, ranging in size from about 5 to 100 cubic inches, were buried in contact with roots of three-year-old rubber trees growing in a sandy loam. The fungicides, which were applied around the collar and tap root and over a six-inch length of the base of the laterals, could not have had any effect on the inocula, which were buried eighteen inches away from the collar. There was a fairly heavy growth of natural covers in the inter-rows, and some grass in the planting rows, where the root sections were buried. These were examined one year after burying, when the results of inoculation were being assessed. The condition of the root sections is summarised in Table 2, which shows that 110 out of 130 were completely disintegrated. In some of the remaining 20 root sections the wood cylinder had entirely disintegrated while the bark retained the outer form; in others there was a soft black or jelly-like rot. In no root section was *F. lignosus* still viable. It is noteworthy that the rotting roots were frequently and extensively penetrated by feeding roots of rubber, sometimes also by the roots of cover plants.

TABLE 2. THE CONDITION OF ROOT SECTIONS INFECTED WITH FOMES LIGNOSUS TWELVE MONTHS AFTER BURIAL

Volume (cu. in.)	Number of roots	No. of roots totally disintegrated	No. of roots with black rot or completely rotted apart from bark
Below 10	26	24 (92%)	2 (8%)
10 – 30	54	46 (85%)	8 (15%)
31 – 50	30	28 (93%)	2 (7%)
51 – 70	10	3 (30%)	7 (70%)
71 – 100	10	9 (90%)	1 (10%)
Total	130	110 (84.6%)	20 (15.4%)

In the third experiment (also designed to test the suitability of various fungicides for the prevention of superficial growth of *F. lignosus* on roots), 120 naturally infected root sections were buried in contact with roots of seven-year-old trees growing in a peaty sand. As in the second experiment, the fungicides which were applied around the collar and tap root, and over a six-inch length of the laterals, could not have had any effect on the inocula, which were buried eighteen inches away from the collar. The natural cover plants in this area had practically died out before the experiment was started. Once again the root sections varied in volume from 5 to 100 cubic inches. They were dug out and examined fifteen months after burial, with the results presented in Table 3, which shows that all except 28 of the 120 roots had completely disintegrated. Of the 28 that remained, only parts could be recovered. In most instances the wood cylinder had disintegrated completely, while the bark kept the form of the root. Both the decaying wood and bark were penetrated by feeding roots of rubber. *F. lignosus* was not viable in any of them.

DISCUSSION AND CONCLUSIONS

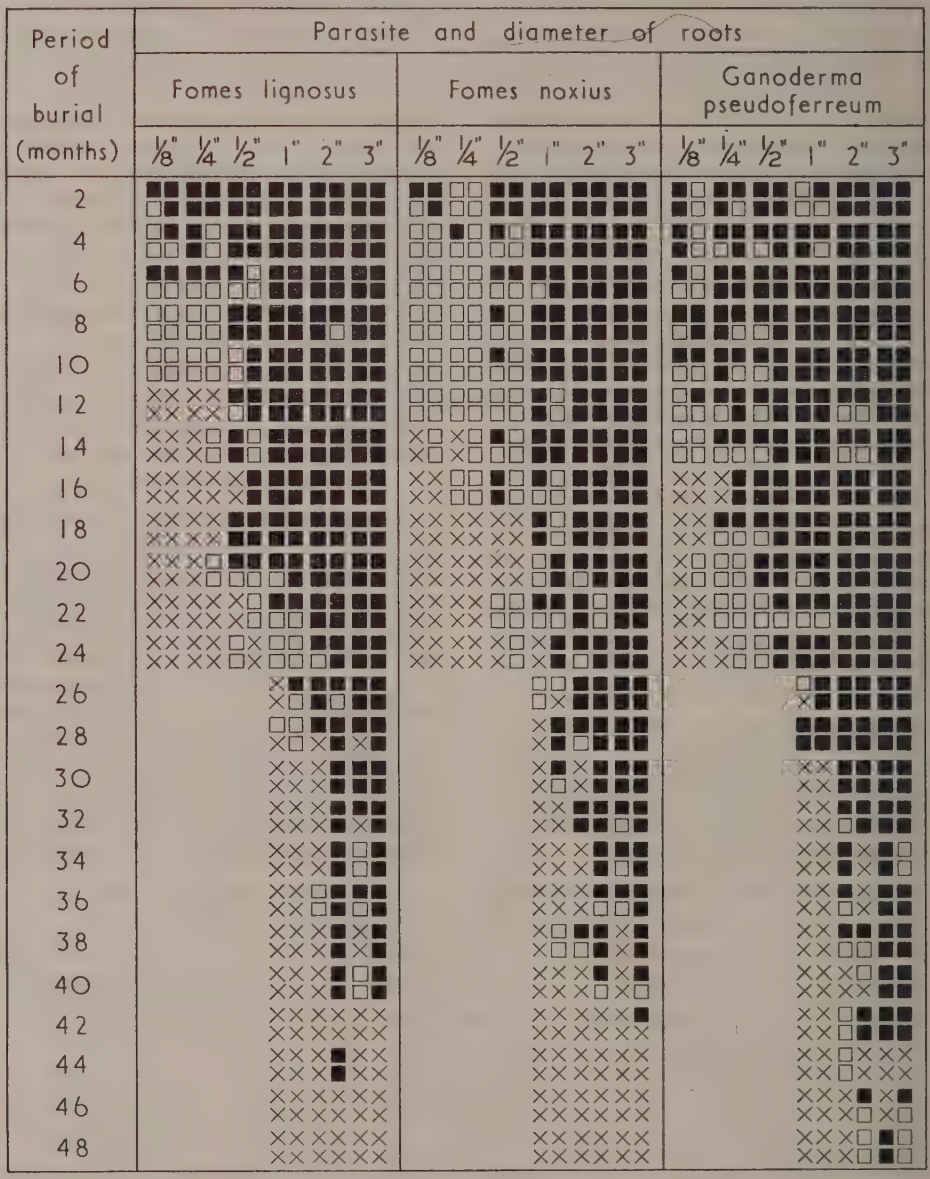
In the burial experiment (Figure 1) the rate of disintegration of infected root sections and the resulting loss of viability of the parasites clearly depended on the size of the root. Here the soil was of low biotic activity, with no living roots and low organic matter content. The root sections used as inocula in the inoculation experiments disintegrated much more quickly, under conditions where the soil was shaded, richer in organic matter, and penetrated by roots. Although an effect of size of the root section on persistence of the parasite could be seen in the third inoculation experiment (Table 3), it was not apparent in the second experiment (Table 2), where loss of viability was more rapid, perhaps because of the heavier ground cover.

There are indications that the feeding roots of rubber assist the disintegration of infected roots, suggesting that the *Hevea* mycorrhiza might be investigated to see whether it is a factor of importance in this regard.

The most significant result of these observations from a practical point of view is the profound effect of soil conditions on the rate of disintegration of infected roots; while the burial experiment showed that the root disease fungi can persist for up to four years in roots three inches in diameter, the inoculation experiments indicate how much more quickly the process of decay can proceed when the area is planted with rubber trees and cover crops. *F. lignosus* was no longer viable in the largest root sections used as inocula after twelve or fifteen months. The inocula must

TABLE 3. THE CONDITION OF ROOT SECTIONS INFECTED WITH FOMES LIGNOSUS FIFTEEN MONTHS AFTER BURIAL

Volume (cu. in.)	Number of roots	No. of roots totally disintegrated	No. of roots with black rot or completely rotted apart from bark
Below 10	20	18 (90%)	2 (10%)
10 – 30	56	48 (86%)	8 (14%)
31 – 50	22	15 (68%)	7 (32%)
51 – 70	10	7 (70%)	3 (30%)
71 – 100	12	4 (33%)	8 (67%)
Total	120	92 (76.6%)	28 (23.4%)



Loss of viability of root parasites in diseased roots; the roots ranged in diameter from ⅛ in. to 3 in. and were either 1 ft or 2 ft in length. The results for each diameter are in pairs: left, length 1 ft; right, length 2 ft.

have ceased to be infective considerably sooner than this. It may be concluded that root fragments remaining after clearing old rubber for replanting are not likely to be more than a passing hazard, even if diseased. The most important consideration is to increase the biological activity of the soil by quickly establishing a good ground cover, so hastening the decay of root fragments.

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The Response of Hevea to Fertilisers on a Sandy Latosol

J. BOLTON

The results of a simple NPK fertiliser trial on Hevea brasiliensis carried out on a sandy latosolic soil are discussed in terms of the response occurring in growth and yield over 10 years of immaturity and the first 12 years of tapping. The main response has been to phosphatic fertilisers with a smaller response to nitrogen but little to potassium. There are some indications that the responses may have been influenced by low magnesium levels in the trees. Clonal sub-plots have given some interesting details of monthly yields throughout the year and yields from different heights of tapping of virgin and renewed bark of five of the older clones. The results emphasise the importance of good growth in the period of immaturity.

There have been few long term fertiliser experiments on *Hevea brasiliensis* reported in the literature, and reports of experiments continued for more than twenty years are particularly rare. The reason for this is not difficult to find when all the hazards which can occur are enumerated and the mass of data which can accumulate is realised. This paper is an account of such a fertiliser experiment carried out in Field 31 at the Experiment Station of the Rubber Research Institute of Malaya which exemplifies the complex nature of even a simply designed experiment on a perennial crop. It is the most accurate and detailed fertiliser experiment carried out on *Hevea* by the Institute since experiments were started in the late 1920's. The volume of data which has accumulated over twenty-four years includes over 90,000 measurements of tree girth and nearly 50,000 measurements of plot latex yield, each of these being cup-coagulated, collected, creped, dried and weighed. Apart from these data, many other supplementary figures have been collected including bark thickness measurements, leaf and latex analyses and soil analyses. Some of this work has been published previously as sections of different papers, but there is a great deal of interesting data which is published here for the first time.

DETAILS OF THE EXPERIMENT

The history of the experiment is most easily set out in tabular form and is given in Table 1. The essential features are the six main fertiliser treatments arranged in a Latin square design and the sub-plots made up of five different clones. The design using only six of the eight fertiliser treatments of a $2 \times 2 \times 2$ NPK factorial layout has already been criticised by Owen *et al.* (1957) as being unorthogonal, and main effects and interactions cannot be estimated by combining treatment means. An estimate can be obtained by making certain assumptions, the main alternatives being to regard K and PK effects as negligible or to assume all the first order interactions to be zero. The former alternative was used in Owen's paper, but the assumption of a zero K effect when there have been many reports of negative K responses in *Hevea* trials (Akhurst and Owen, 1950) seems to be questionable. The second alternative also seems inadvisable as significant positive NK interactions were found by Owen *et al.* in the 17 trials reported in their paper. No valid assumptions can therefore be made to enable combinations of treatment means to give the effects of the main fertilisers but, fortunately, a low coefficient of variance (probably due to the large plots and

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TABLE 1. DETAILS OF THE EXPERIMENT

Design	6 × 6 Latin Square
Fertiliser treatments	Control, N, NP, NK, PK, NPK
Sub-plots	Planted with Tjir 1, PB 86, Pil B84, Pil D65, BD 5
Planted	December 1935 with unselected pencil stump seedlings
Budded	November 1937
Plot size	1.08 acres, with 194 tree points per plot, divided into 5 clonal sub-plots each of 39 points
Planting distance	22 × 11 ft on contour terraces
Covers	Natural. Controlled by periodic slashing
Tapping system	Full spiral, 4th daily, S/1.d/4.100%
Tapping panels	1st Panel—opened at 36 in. above the union, August 1947. Lowest part tapped out half spiral alternate daily, S/2.d/2.100%, during 6 months in 1953
	2nd Panel—opened at 41 in. above the union October 1953 thus creating a virgin bark island. Below the virgin bark island, tapping was on first renewed bark.

latin square design) enables each treatment mean to be considered individually and this procedure will be followed in the following account.

Topography

The area is not ideal for experimental purposes being on the western slopes of a quartzite hill. Several deep gullies run down the hill towards a comparatively level low-lying flat area, which is, however, reasonably well drained. The whole area was planted on terraces built on level contours approximately 22 ft apart and the plots were laid out on a superimposed grid which kept all the plot and sub-plot boundaries straight. Care was taken at planting and at each thinning to ensure a uniform tree density throughout the experiment. This method has been found quite satisfactory in practice and has been subsequently used in experiments on several other hillside sites.

Soil

The soil, a sandy loam, was derived initially from quartzite which weathers at an early stage to sandstone. It has been described by Owen (1951) as of the Kedah Series, although, in subsequent more detailed studies of similar soils, Panton (1954) reserves this name for a more eroded phase of quartzite-derived soils. The latter author's description of the Serdang Series is more similar to that found in the experimental area. Mechanical analyses of samples taken in 1951 from the terraces and from under the covers of the control plots are given in Table 2. Additional soil analyses of samples taken during 1959 from all the plots are discussed in detail in a later section.

TABLE 2. ANALYSES OF SOIL SAMPLES (0–6 INCHES) TAKEN IN 1951 FROM THE CONTROL PLOTS. THE RESULTS ARE QUOTED AS PERCENTAGES OF AIR DRIED SOIL

Sampling area	Gravel	Sand		Silt	Clay	Loss on Ignition	pH	N	C
		Coarse	Fine						
Terraces	Nil	41.6	41.4	3.5	10.9	3.39	5.18	0.067	0.91
Under cover	Nil	42.1	39.4	4.0	10.9	4.70	5.02	0.108	1.46

TABLE 3. SCHEDULE OF FERTILISER APPLICATIONS (OZ PER TREE)

	Ammonium sulphate	Rock phosphate	Double superphosphate	Potassium	
				chloride	sulphate
Immature					
April 1936	Basic dressing				
May 1937	3½	—	2	—	2
November 1937	Budded				
May 1938 and Oct. 1938	3½	—	2	—	2
April 1929 and Nov. 1939	5	—	2.5	—	2.5
April 1940	10	—	5	4	—
April 1941	12	—	6.25	5	—
Mature					
July 1947 and biennially	40	16	—	8	—

Manuring

A programme of manuring was followed from planting in 1936 up to the end of 1941 when, owing to the war, treatments had to be stopped (Table 3). In the pre-war treatments, soluble phosphates were used and the ratios of $N : P_2O_5 : K_2O$ were 1 : 1 : 1.2, while in later years when the trees were in tapping rock phosphate was used and the ratios were 1 : 0.7 : 0.6. The post-war proportion of potassium was presumably reduced when the results showed no beneficial effects from this nutrient.

Since 1947, fertilisers have been applied biennially, usually in the May–July period. The rates of these applications are also shown in Table 3.

All the fertilisers were applied on the contour terraces which have been kept clean of weeds. Dressings of ammonium sulphate and potassium chloride were broadcast while from 1947 the rock-phosphate fertiliser was applied in 'pockets' around each tree, but this practice was discontinued in 1957–58 when this fertiliser was also broadcast. The effect of this method of application on the results of soil analyses on each plot is discussed further below.

Recording

Pre-war girth measurements and two sets of measurements in 1947 were made at 50 inches above the union of stock and scion; all others, including the annual measurements since 1947, were made at 60 inches.

Latex yield measurements were started on opening the trees for tapping in 1947 using a cup-coagulation technique. After collection, the cup lumps from each sub-plot were washed, creped and dried in the smoke-house for ten days, then weighed. Recordings were made on two tapping days per month from opening in 1947 until 1955, and on one tapping day thereafter.

Soil samples were collected from the terraces of each plot in 1959, leaf samples (from the Tjir 1 trees only) in 1954, and latex samples from three clones in 1957. In 1950 the thickness of both virgin and first renewed bark was also measured using a Schlieper

gauge. The construction and use of this instrument has been described previously by de Jonge (1957).

RESULTS

In order to avoid the presentation of extensive tables of girth, girth increments and yields for both the different fertiliser treatments and the five different clones, most of the data have been summarised in graphic form. In this way, the main feature of the results can be more easily seen but less prominence is given to the standard errors and statistical significance of the results, although these have been indicated wherever possible.

Effect of Fertilisers on Growth

The effects of the different fertilisers on growth in the immature stage have been briefly discussed, along with the results of other experiments, by Akhurst and Owen (1950). There was a marked response to soluble phosphate fertilisers with a smaller response to nitrogen, but little evidence of any additional benefit from potassium. Figure 1 shows the mean growth curves for the control and NPK plots over the 22-year period from budding to mid-1959.

The best growth up to the sixth year was in the NP plots (omitted from the graph for the sake of clarity), but in the 1943–46 period (when no further fertilisers were applied) these were overtaken by the NPK plots. From 1945 to 1947 there was less than 2% difference in growth (non-significant) between all six treatments, which suggests that the residual effect of the earlier fertilisers had died out.

After fertiliser dressings were resumed in 1947, significant differences in annual growth

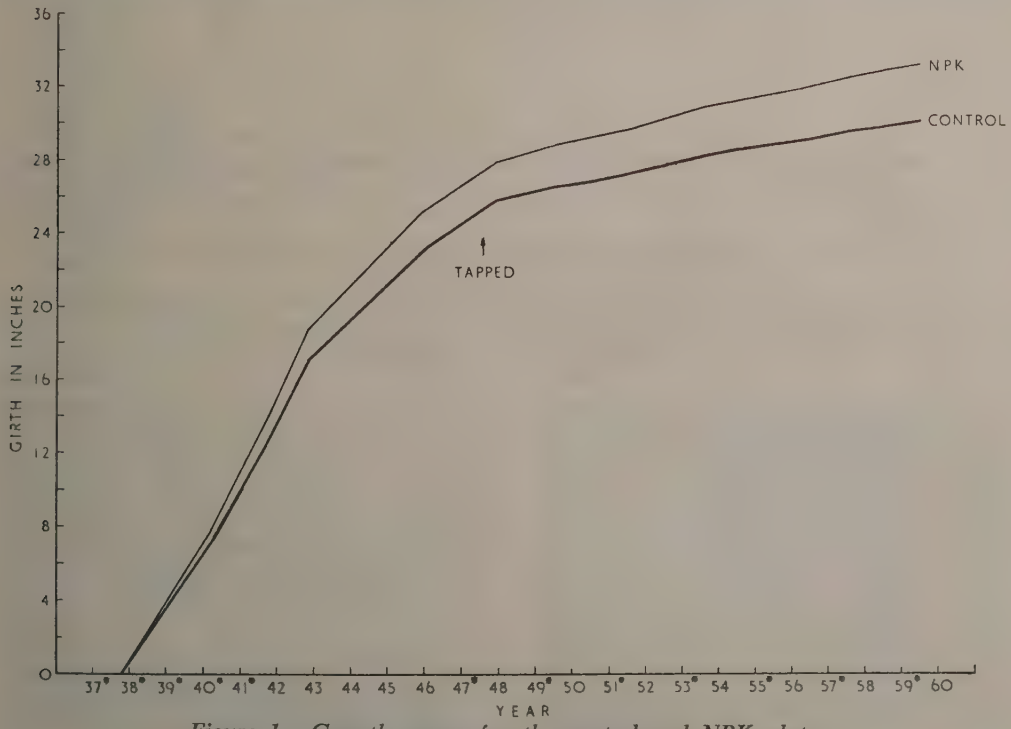

Figure 1. Growth curves for the control and NPK plots.
** Year of fertiliser application.*

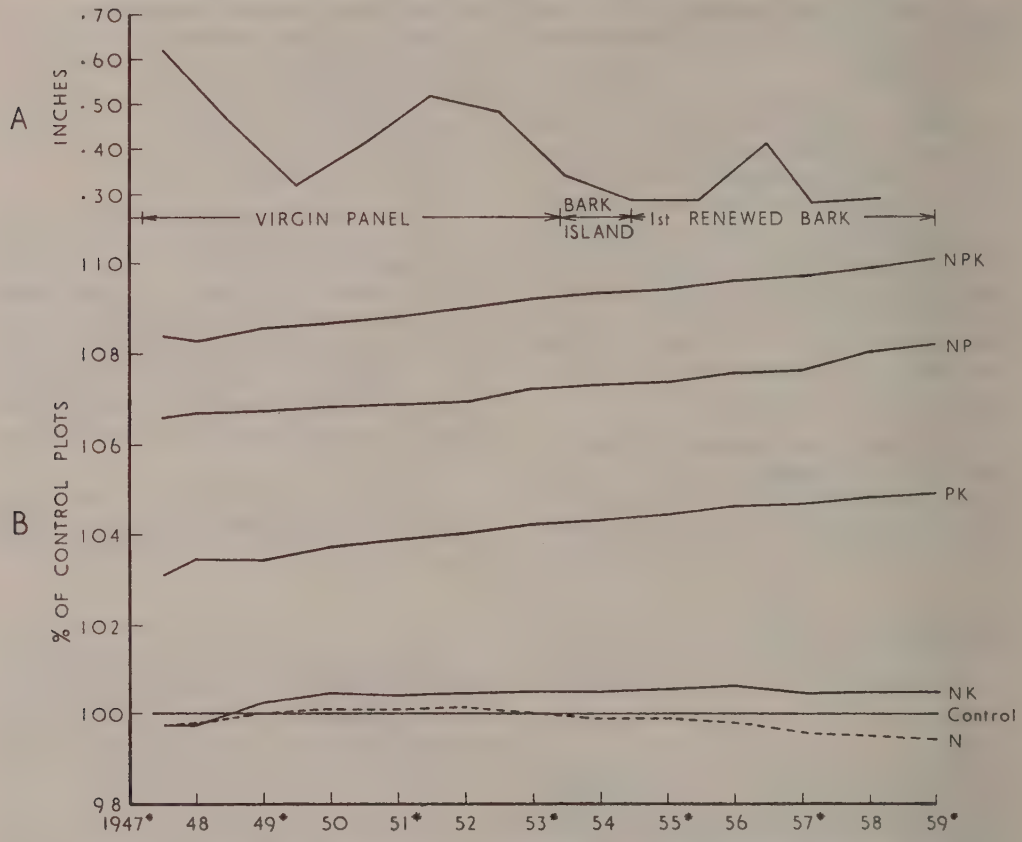

Figure 2. Girth, as a percentage of the control, for each fertiliser treatment (B), together with the annual girth increments of the control plots (A).
** Year of fertiliser application.*

were again measured and these are illustrated in Figure 2(B) where the mean girths for the different treatments are given as a percentage of the control plots. Growth in the NPK plots was slightly better than in the NP plots but in no year was the difference between them statistically significant. Potassium has had therefore a very minor, if any effect on growth. Nitrogen too seems to have had little effect on growth after maturity, the growth curves for the NPK and the PK plots being almost parallel. In the absence of P and K however, the effect of the nitrogen fertiliser had a definite trend from slightly beneficial in 1948 to a deleterious effect, particularly in the 1956–59 period, although in no year were the differences statistically significant from the control. This trend might be explained in terms of the effect of sulphate of ammonia on the magnesium nutrition of the trees and is discussed further below in relation to the leaf and soil analyses.

The annual girth increments of the control plots (Fig. 2A) are interesting in that they show regular differences as the tapping cut moves down the panel on both virgin and renewed bark.

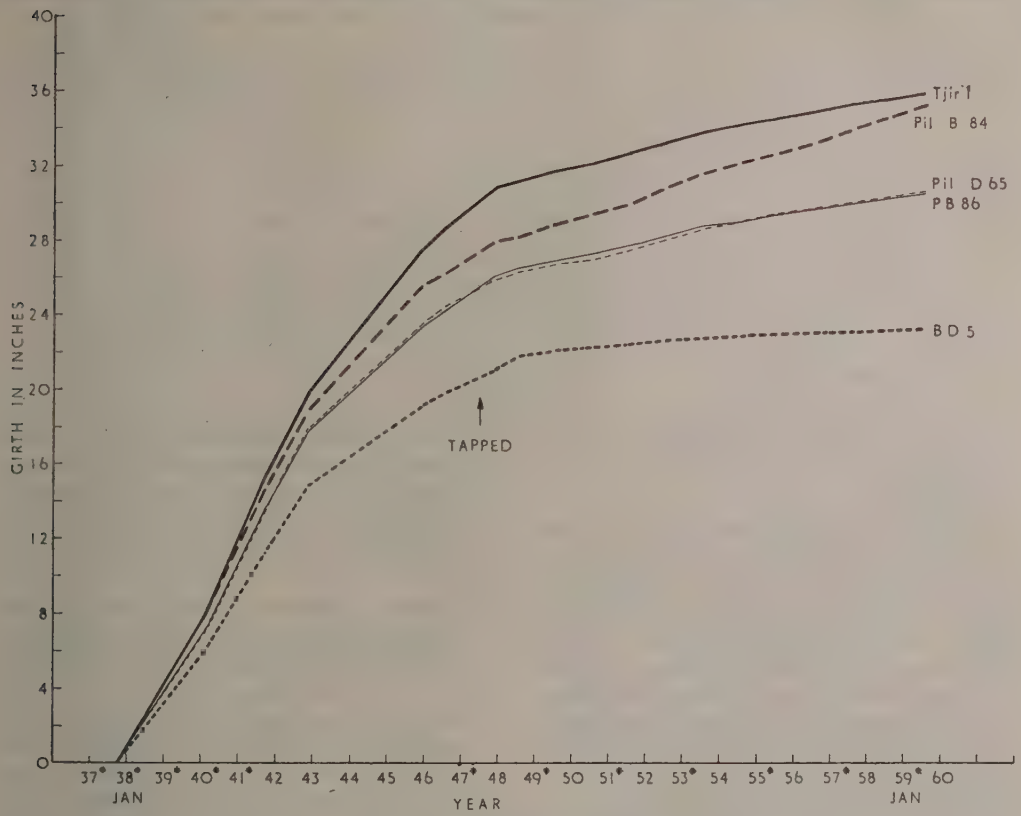

Figure 3. Growth curves for each of the five clones.
** Year of fertiliser application.*

There was no evidence of any differential clone reaction to the fertilisers.

Clonal Differences in Growth

Growth curves for the five clones are shown in Figure 3. Differences in the form of the curves both before and after tapping are readily apparent and call for little further comment. The more prominent features are the very poor growth of the clone BD 5 after tapping, when the girth increased by only 1.7 inches in a period of 11 years, and the comparatively good growth of Pil B84 which grew at a rate almost double that of PB 86, Pil D65 and Tjir 1 after opening. The poor growth of BD 5 was apparent from the first girth measurements taken 2½ years after budding.

The Effect of Fertilisers on Yield

Of more direct interest to the industry is the effect of continued application of fertilisers on yield. In this experiment, the yield responses have closely followed the responses in girth but have been much more pronounced. This can be seen in Table 4 where the girth and yields for the different fertiliser treatments are set out as percentages of the control plots at the beginning and the end of the tapping period. These figures show that the increases in yield due to the treatments cannot be fully explained solely by increased

TABLE 4. GIRTH-YIELD RELATIONSHIP GIVEN AS A PERCENTAGE OF CONTROL PLOTS

		O	N	NK	NP	PK	NPK	s.e.±	L.S.D.*
1947	Girth	100	101	100	107	103	108	1.5	4.3
	Yield	100	104	105	106	109	116	3.1	9.2
1959	Girth	100	99	100	108	105	110	1.3	4.0
	Yield	100	96	97	121	116	129	5.2	15.3

* $p = 0.05$

girth and the consequently greater length of the tapping cut, i.e. by a linear girth-yield relationship.

Correlations between girth and yield have been made at several times during the life of the experiment. The first was in 1947 when highly significant correlations were found within the different classifications of the experiment, i.e. between clones, between manurial treatments and between main plots. It was suggested from these measurements that the true regression is curved in the sense that larger trees yield more per inch of the tapping cut than the smaller trees.

In 1949 further calculations were made and again a highly significant correlation ($r = +0.475$) was obtained. Similar calculations carried out subsequently on the 1949 plot data supported the view that the true regression was curved and a log girth—log yield regression gave a highly significant correlation ($r = +0.663$) and a relationship of the form yield $\propto$ girth^M where $M = 1.2$. Data from other areas give figures nearer $M = 1.5$ (PAARDEKOOPER, 1960).

There is no obvious explanation of the cause of this relationship. It was thought that perhaps the larger trees had thicker bark and that this might be the cause of the curvature, but measurements taken in 1950 showed no significant differences between the bark thickness of the different main plots for either virgin or renewed bark (Table 5). There were however, marked differences in bark thickness between the five clones. Further investigation of girth-yield relationships are required, on both individual trees and on differently treated plots.

Figure 4 shows the yield for the different treatments as a percentage of the control plots. All the phosphate treated plots showed an increase in yield compared with the controls, whereas, as for the growth rate, there was a tendency for yield in the nitrogen treated plots to decrease. If the curves for NPK and NP are smoothed out, it is found that the rate of increase of both treatments over the control is the same, showing that K has had a small and non-significant positive effect in the presence of the other two nutrients.

The rate of increase of both these curves over the control is about 1.25% per annum. The significance of this figure is discussed further below, in relation to the costs of the fertiliser applications and the effects of the fertiliser applied during immaturity.

Clonal Differences in Yield

The mean yield for all five clones was surprisingly uniform throughout the 12-year period, the only fall being in 1953 when the tapping system was changed to S/2.d/2 for a few months, after which a new panel was opened 6 inches above the old panel and a bark 'island' created (Figure 4).

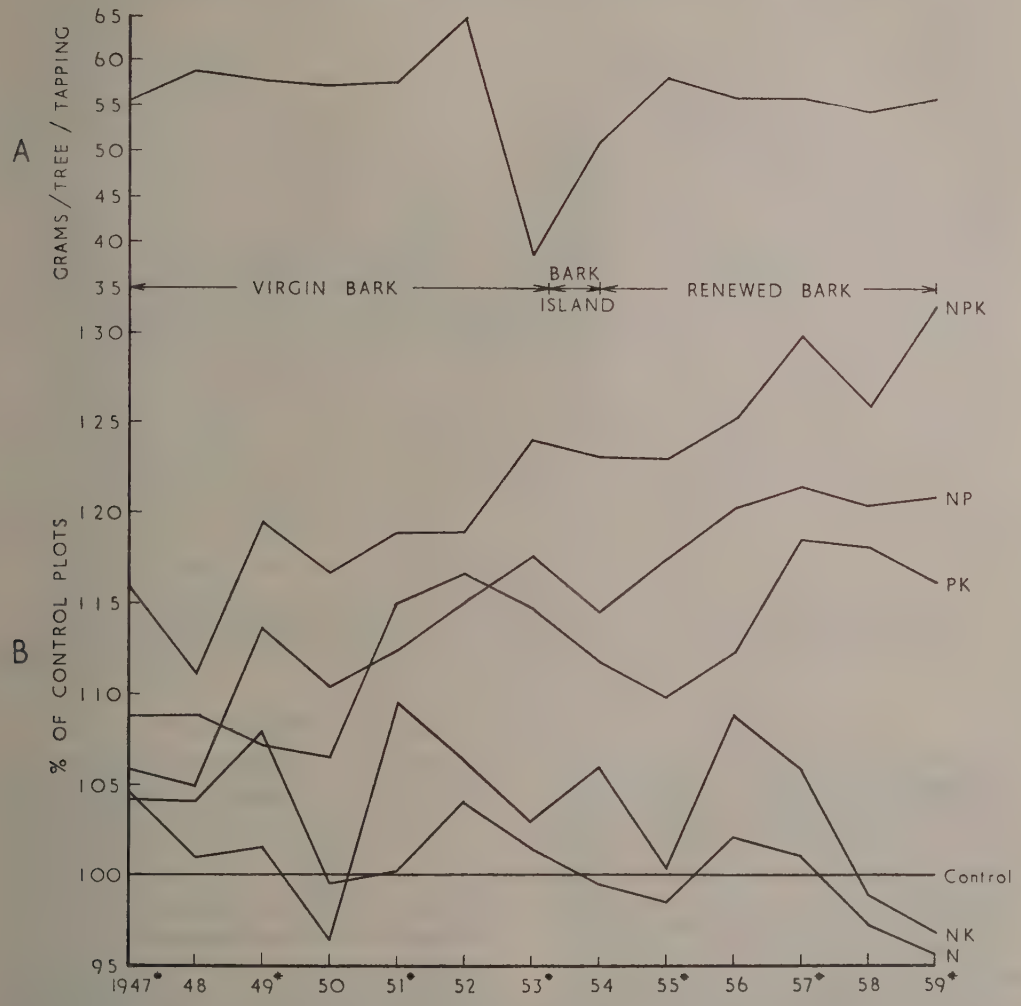

Figure 4. Annual yield, as a percentage of the control, for each fertiliser treatment (B) together with the annual yields of the control plots (A)
** Year of fertiliser application.*

Yields from the first renewed bark were slightly less than for virgin bark but the difference was only about 4%, when averaged for all the clones.

(a) *Annual yields.* A graph of the annual yields for each clone is given in Figure 5, and this shows quite clearly the different reaction of each clone to tapping. Tjir 1 gave an increased yield as the cut approached the union on both virgin and renewed bark. The other clones however showed a slight drop in yield after the first two years and then a sharp increase nearer the union. The poorest clone, BD 5, showed a 30% decrease in yield when tapped on first renewed bark and, in 1959, could not be tapped again on the lower

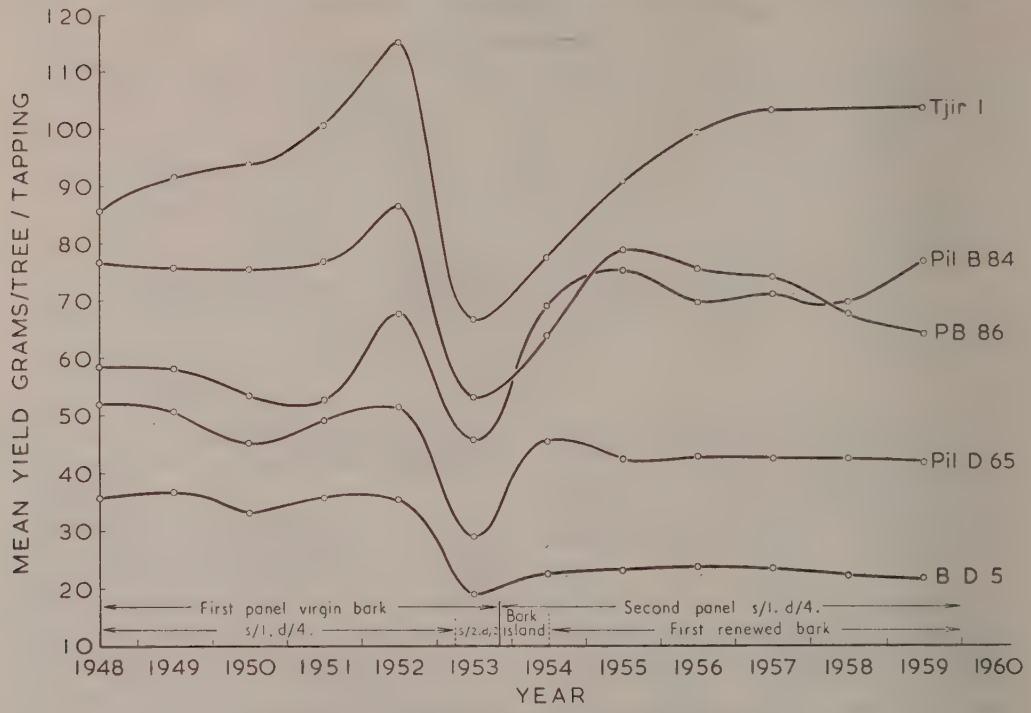

Figure 5. Annual yields for the five clones.

panel because of the very poor bark renewal. A surprising feature of the Pil B84 curve is the higher yield on renewed bark than on virgin bark. This may be a consequence of the outstanding growth of this clone whilst in tapping.

(b) *Monthly yields.* The mean yield of the different clones month by month is of some interest (Figure 6). The graph shows the different response to wintering for each of the five clones, Tjir 1 having a 38% reduction in yield as compared with a reduction of 22% for PB 86. The Tjir 1 also has a different shape of yield curve after wintering, there being a steady increase in yield from June to December. The other clones however gave more uniform yields in the same period, although there was a tendency for yields to rise in the months of November, December and January. The slight drop in yield for some of the clones in the August–October period is also interesting but no satisfactory explanation can be given.

No simple relationship was found between yield and rainfall on a long or short term basis.

LEAF, LATEX AND BARK ANALYSES

Leaf samples were taken together with bark samples from the Tjir 1 trees in 1953 and have been fully reported by BOLLE-JONES (1957). Standards for satisfactory levels of nutrients in the leaf laminae have been formulated since this work was reported and on the basis of these the area would be considered as adequately supplied with potassium, slightly deficient in nitrogen and magnesium and very low in phosphate. This agrees substantially with the responses which have occurred. The phosphate level in the phosphate-treated plots was also low by the

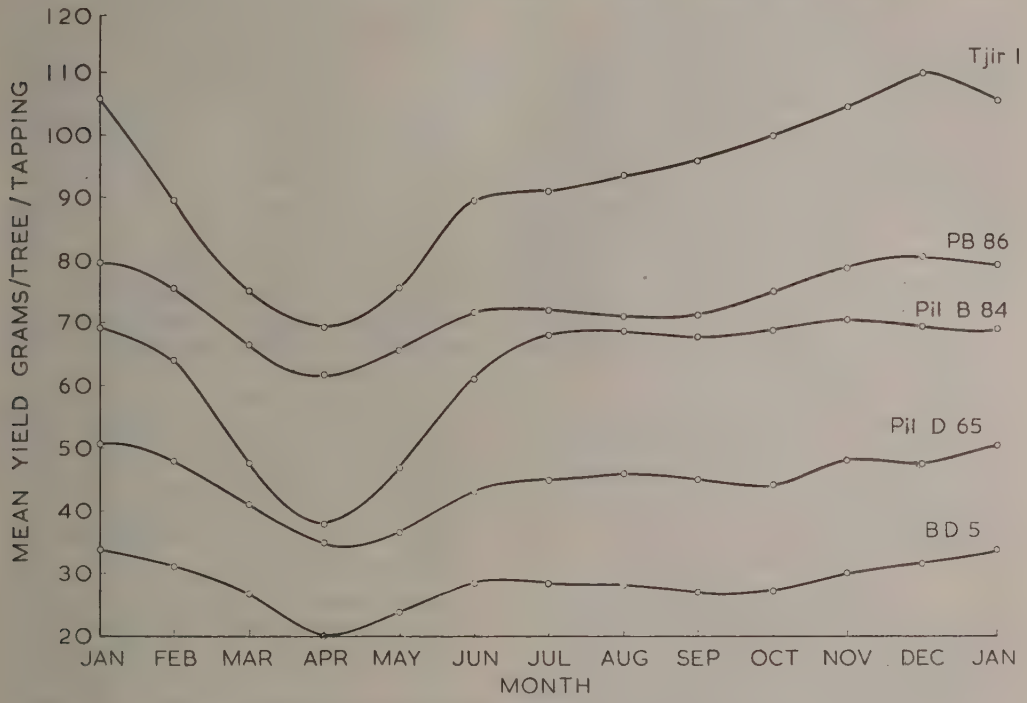

Figure 6. Mean monthly yields for the five clones.

standards now used, which suggests that the amounts of rock phosphate used were too low for optimum growth and yield.

Samples of latex were collected from three clones in 1957 and the analyses were compared with standards published by Beaufils (1957). Assessed solely on the latex analyses, the trees would be considered adequately supplied with potassium but sub-optimum in nitrogen and magnesium. Phosphate was below optimum for two clones Tjir 1 and PB 86 but not for Pil B84, although phosphate responses have been observed on all three clones. Details of the leaf and latex analyses, together with the standards used, are given in Table 5.

SOIL ANALYSES

In 1959, soil samples were taken from the terraces of each plot, these being the areas where all the fertilisers had been applied. The results are summarised with the other analyses in Table 5. The main effects observed were large increases in exchangeable potassium and available phosphate due to the potassium chloride and rock phosphate applications, and highly significant depressions in pH due to the ammonium sulphate. As would be expected, there were also increases in exchangeable calcium due to the rock phosphate applications.

Owen (1953) has suggested that a response to phosphate should be obtained at levels of available phosphate in the soil below 25 p.p.m. (using the same analytical method as used in these studies). Values of 8–9 p.p.m. would therefore indicate a severe deficiency, which has been confirmed by the large growth and yield responses obtained. The phosphate analyses were also rather variable owing to the method of application previously mentioned. The figures for untreated plots ranged from 2–15 p.p.m., whereas the phosphate treated plots gave a range of from 7 to

TABLE 5. LEAF, LATEX AND SOIL ANALYSES FOR EACH FERTILISER TREATMENT TOGETHER WITH BARK THICKNESS MEASUREMENTS

Treatment	Leaf analyses Tjir 1 (1953)						Soil analyses (1959)				
	N	P	K	Mg	Ca	Mn	pH	Av. P_2O_5	Exchangeable K	Mg	Ca
	percentage of dry matter					p.p.m.		p.p.m.	m-equiv./100g		
O	3.19	0.15	1.18	0.23	0.49	114	4.93	8.8	0.047	0.043	0.057
N	3.02	0.15	0.97	0.19	0.58	93	4.46	8.0	0.040	0.041	0.051
NK	3.27	0.15	1.20	0.18	0.38	56	4.58	7.8	0.056	0.045	0.051
NP	3.27	0.18	1.19	0.22	0.39	77	4.34	25.1	0.049	0.044	0.059
PK	3.15	0.18	1.08	0.22	0.49	76	5.06	38.0	0.080	0.045	0.071
NPK	3.11	0.18	1.15	0.20	0.46	104	4.46	10.7	0.077	0.051	0.066
s.e.	0.09	0.01	0.10	0.01	0.06	25	0.07	14.9	0.012	0.04	0.005
L.S.D.*	0.27	0.03	0.30	0.05	0.19	75	0.21	43.9	0.035	0.11	0.016
Standard	3.25	0.20	0.80	0.20	0.40	50	—	25	0.035	0.110	—

Treatment	Latex analyses (1957)					Bark thickness (1950)	
	N	P	K	Mg	Mg/P	Virgin	Renewed
	percentage of total solids					mm	mm
O	0.573	0.096	0.397	0.070	0.73	8.37	6.49
N	0.581	0.090	0.387	0.069	0.77	8.27	6.47
NK	0.575	0.096	0.402	0.070	0.74	8.43	6.40
NP	0.603	0.122	0.386	0.075	0.61	8.29	6.48
PK	0.589	0.126	0.427	0.066	0.53	8.35	6.50
NPK	0.575	0.114	0.412	0.070	0.61	8.58	6.56
s.e.	0.007	0.004	0.011	0.004	0.04	0.11	0.09
L.S.D.*	0.019	0.011	0.033	0.011	0.13	0.34	0.28
Tjir 1	0.590	0.101	0.374	0.077	0.79	9.02	7.13
PB 86	0.562	0.098	0.405	0.080	0.85	8.27	6.37
Pil B84	0.595	0.122	0.427	0.052	0.43	9.12	7.37
Pil D65	—	—	—	—	—	8.13	5.83
BD 5	—	—	—	—	—	7.36	5.72
s.e.	0.006	0.003	0.011	0.003	0.03	0.11	0.10
L.S.D.*	0.019	0.008	0.035	0.008	0.09	0.32	0.29
Standards					0.71–1.26	—	—

* $p = 0.05$

229 p.p.m. Obviously the highest figures are due to a number of phosphate 'pockets' being sampled.

BOLTON (1961) has tentatively suggested threshold levels of magnesium (0.11 m–equiv/100g) and potassium (0.035 m–equiv/100g) and on this basis the control plots would be considered magnesium deficient but above the 'deficiency' level in potassium.

Reductions of exchangeable calcium, magnesium and potassium associated with the lowering of pH in the nitrogen plots were not statistically significant. However, subsequent lysimeter experiments carried out by BOLTON (1960) on the same soil type have shown definite reductions to occur. Such depressions in pH and exchangeable cations resulting from applications of ammonium sulphate are reported by the same author (1961) in several other field experiments on *Hevea in* Malaya.

The pH measurements showed that there was a gradient over the field, the lower slopes having higher pH values than the upper slopes.

DISCUSSION

Comparing the effects of fertiliser applications, before and after maturity, it is obvious that by far the greatest return is from the former dressings. In total amount, the fertilisers applied from planting to four years after budding were equivalent to just over 2½ lb of ammonium sulphate, 1½ lb of concentrated superphosphate and 1 lb of potassium chloride per tree and this resulted in an initial yield response of about 14%. After maturity, *each* biennial application of fertilisers at 2½ lb ammonium sulphate, 1 lb rock phosphate and ½ lb of potassium chloride (approximately the same amount) resulted in a mean increase in yield of only 2.5% (assuming no residual effects from the pre-war fertilisers).

In using these figures to calculate the economics of the fertiliser applications, it must be assumed that the total response is the sum of the response to the pre-war and post-war applications and that the latter can be divided into equal responses for each dressing. If these assumptions are accepted then a 2.5% increase in annual yield from the time of each post-war fertiliser application will easily pay the costs at present prices of fertilisers and dry rubber (disregarding the value of residual effects which almost certainly occur). The conditions in the experiment are not however directly comparable with commercial conditions in that all the trees were opened for tapping at the same time. Normally, the trees would be opened at a fixed girth—usually 20 in.—and there would merely be a time interval between the opening of different plots.

This experiment does emphasise the importance of adequate care and fertiliser dressings during immaturity. If girth is the main determining factor for yield from any particular clone then it is far easier to increase the girth during immaturity when the trees are growing at a rate of four to five inches per annum than after tapping when the girth increments are reduced to less than one inch.

The rate of yield increase of the NP plots over the control has been just as great as for the NPK plots, the potash seemingly having little or no extra effect. Ammonium sulphate alone, as has already been mentioned, tended to depress both growth and yields and this effect is rather difficult to explain solely on the data from the trial. It may have been due to the effect of the fertiliser on soil, leaf and latex magnesium, since these were all rather low, but there is no direct evidence of this The use of ammonium sulphate by itself on young mature trees does however seem questionable, at least where other nutrients such as magnesium or potassium are likely to be sub-optimal. The results obtained from potassium fertiliser may also have been influenced by the low levels of magnesium and this may be an important point when translating these results into specific recommendations to planters. The low level of available magnesium, as well as the unsatisfactory design, has severely limited the utility of the trial and emphasises the care which should be taken in establishing long term trials with perennial crops, which take up so much time and labour over a period of many years.

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